EXHIBIT 2

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Structure of the *N*-Linked Oligosaccharides That Show the Complete Loss of α-1,6-Polymannose Outer Chain from *och1*, *och1 mnn1*, and *och1 mnn1 alg3* Mutants of *Saccharomyces cerevisiae**

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The periplasmic invertase was purified from *Saccharomyces cerevisiae och1::LEU2* disruptant cells (Δ*och1*), which have a defect in elongation of the outer chain attached to the *N*-linked core oligosaccharides (Nakayama, K., Nagasu, T., Shimma, Y., Kuromatsu, J., and Jigami, Y. (1992) *EMBO J.* 11, 2511-2519). Structural analysis of the pyridylaminated (PA) neutral oligosaccharides released by hydrazinolysis and *N*-acetylation confirmed that the *och1* mutation causes a complete loss of the α-1,6-polymannose outer chain, although the PA oligosaccharides ($Man_8GlcNAc_2$-PA and $Man_{10}GlcNAc_2$-PA), in which one or two α-1,3-linked mannose(s) attached to the endoplasmic reticulumn (ER)-form core oligosaccharide ($Man_8GlcNAc_2$) were also detected. Analysis of the Δ*och1 mnn1* strain oligosaccharides released from total cell mannoprotein revealed that the Δ*och1 mnn1* mutant eliminates the α-1,3-mannose attached to the core and accumulates predominantly a single ER-form oligosaccharide species ($Man_8GlcNAc_2$), suggesting a potential use of this strain as a host cell to produce glycoproteins containing mammalian high mannose type oligosaccharides. The Δ*och1 mnn1 alg3* mutants accumulated $Man_5GlcNAc_2$ and $Man_8GlcNAc_2$ in total cell mannoprotein, confirming the lack of outer chain addition to the incomplete corelike oligosaccharide and the leaky phenotype of the *alg3* mutation. All the results suggest that the *OCH1* gene encodes an α-1,6-mannosyltransferase that is functional in the initiation of α-1,6-polymannose outer chain addition to the *N*-linked core oligosaccharide ($Man_5GlcNAc_2$ and $Man_8GlcNAc_2$) in yeast.

We have described new temperature-sensitive (*ts*) *Saccharomyces cerevisiae* mutants, which exhibit a deficiency in mannose outer chain addition of asparagine (*N*)-linked oligosaccharides (1). The strains, designated *och1* and *och2*, were obtained by the [^{3}H]mannose suicide technique, and the *och1* mutant showed a 2:2 cosegregation with regard to the glycosylation defect and *ts* growth phenotype. The size of external invertase of the *och1* mutant was slightly larger than that of the *sec18* mutant, which accumulates the ER-form core oligosaccharide ($Man_8GlcNAc_2$) at the non-permissive temperature (2), but it was slightly smaller than that of *mnn9* mutant, which contains two mannoses of outer chain attached to the core oligosaccharide (3). Cloning of the *OCH1* gene, by complementation of *ts* growth phenotype, and subsequent characterization of the gene product demonstrated that the *OCH1* gene encodes a novel membrane bound mannosyltransferase (4). The OCH1 protein-overproducing cells exhibited about 5-fold greater mannose transfer activity with the mannoprotein prepared from the *och1::LEU2* disruptant cells (Δ*och1*) as an acceptor for the *in vitro* mannose transfer reaction, while the smaller oligosaccharides, such as α-methyl-mannoside, α-1,6-mannobiose, and mannotetraose, used to measure conventional α-1,2- or α-1,6-mannosyl transfer, were inactive.

Therefore, further studies on the structure of acceptor molecule for the OCH1 protein-dependent mannose transfer are required to elucidate the role of OCH1 protein in outer chain biosynthesis in yeast. Here we report the structures of *N*-linked oligosaccharides of external invertase prepared from Δ*och1* cells and those of cell wall mannoprotein prepared from Δ*och1 mnn1* double mutant and from Δ*och1 mnn1 alg3* triple mutant. The structural relationship indicated that the strains containing the Δ*och1* mutation completely lack the α-1,6-polymannose outer chain attached to the core oligosaccharide ($Man_8GlcNAc_2$), not only in a specific glycoprotein, invertase, but also more generally in cell wall mannoprotein. The accumulation of predominantly a single oligosaccharide, $Man_8GlcNAc_2$, due to the disappearance of α-1,3-linked mannose addition, was also confirmed in the Δ*och1 mnn1* mutant. Together with previous results on the properties of the OCH1 protein (4), the results reported herein indicate that the *OCH1* gene encodes a membrane-bound α-1,6-mannosyltransferase that is functional in the initiation of the polymannose outer chain addition to the *N*-linked core oligosaccharide.

EXPERIMENTAL PROCEDURES

Strains and Media

S. cerevisiae strain EHA-1C (*MATa leu2-3 leu2-112 pep4-3*) has been described in a previous paper (1). *S. cerevisiae* YS52-1-1B, in which the *OCH1* gene is disrupted by *LEU2* has been described previously (4). *S. cerevisiae* YS57-5A (*MATa och1::LEU2 leu2 ura3 his1 his3*), YS57-5C (*MATa och1::LEU2 leu2 ura3 trp1 his1 his3*), and YS57-2C (*MATa och1::LEU2 leu2 ura3 trp1 his1 his3*) were constructed by the standard genetic methods (5) as described (4). *S. cerevisiae* LB1-10B (*MATa mnn1*) (6) was obtained from the Yeast Genetic Stock Center, University of California, Berkeley. *S. cerevisiae* LB347-1C (*MATa mnn9*) (7), was provided by Dr. W. Tanner (University of Regensburg, Germany). *S. cerevisiae* PRY90 (*MATa alg3-1 ade2-101 ura3-52*) (8) was provided by Dr. P. W. Robbins, Massachusetts Institute of Technology. Cells were grown in YPD medium (2% Bacto-peptone, 1% yeast extract, 2% glucose), supplemented with 0.3 M sorbitol as an osmotic stabilizer.

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Purification of Invertase

YS52-1-1B (Δ*och1*) was grown in a 5-liter jar fermenter containing 3 liters of medium (YPD + 0.3 M sorbitol) at 25 °C. When glucose was exhausted by the cells, 0.5% sucrose was added to de-repress the invertase gene expression. The cells were incubated for an additional 4 h and then harvested by centrifugation. Invertase activity was assayed at 30 °C by measuring the rate of glucose released from sucrose with Glucose CII-Test Wako (Wako Chemical Co., Osaka, Japan) in sodium acetate buffer (pH 5.0) according to the supplier's manual.

External (periplasmic) invertase was purified by Neumann and Lampen (9) with some modifications. All operations were done at 4 °C. The cells obtained from 12-liter cultures were suspended in 10 mM potassium phosphate buffer (pH 6.5) (buffer A) containing 1 mM phenylmethylsulfonyl fluoride and broken in a French pressure cell press (SLM, Aminco) at 12,000 p.s.i., and the homogenates were centrifuged to remove the cell debris. Streptomycin sulfate (1% final) was added to remove nucleic acids. Ammonium sulfate was then added to the supernatant until 75% saturation to precipitate nonglycosylated internal invertase and most of the contaminating proteins. After centrifugation, the supernatant was dialyzed against the buffer A. Ten grams of DEAE-Sephadex A-50 resin, preequilibrated with buffer A, was added to the dialysate. After the invertase activity remaining in solution was reduced to less than 10% of the initial, the liquid was removed, and the resin was packed in a column (2.6 × 35 cm) and washed with two volumes of the same buffer containing 0.1 M NaCl. External glycosylated invertase was then eluted with the buffer containing 0.2 M NaCl, and the fractions containing enzyme activity were pooled and dialyzed against deionized water. The dialysate was lyophilized, dissolved in deionized water, and applied to a Sephadex G-200SF column (2.0 × 95 cm) equilibrated with buffer A containing 0.2 M NaCl. The column was eluted with the same buffer, and the invertase-containing fractions were pooled and lyophilized. The sample thus obtained gave a discrete protein band on SDS-PAGE[1] (10) using a 4–20% gradient gel (SDS-PAGE Plate 4/20, 10 cm × 10 cm, Daiichi Pure Chemicals, Tokyo, Japan) by Coomassie Brilliant Blue staining. Table I summarizes the purification of external invertase from Δ*och1* cells (YS52-1-1B strain) obtained from a 12-liter culture. The yield of purified glycosylated invertase was 2 mg from 180 g of wet cells. The above process was repeated four times to obtain sufficient invertase to analyze its oligosaccharide structure.

Hydrazinolysis and N-Acetylation

About 8 mg of purified external invertase, isolated from Δ*och1* cells according to the above procedure, was subjected to hydrazinolysis to release *N*-linked oligosaccharides (13, 14), using a gas-phase hydrazinolysis apparatus (Hydraclub S-204, Honen Oil Co., Tokyo, Japan). The glycoprotein samples (0.5 mg/vial) were treated for 60 min at 110 °C. After hydrazinolysis, the oligosaccharides were *N*-acetylated at room temperature for 30 min by adding 250 μl of 0.2 M ammonium acetate and 25 μl of acetic anhydride, and this process was repeated once to complete the reaction.

Preparation of Mannoprotein

Cells were cultivated in YPD-sorbitol medium at 25 °C (YN3-1D, Δ*och1 mnn1*) or at 28 °C (YN5-2C, Δ*och1 mnn1 alg3*) and harvested at early stationary phase. Mannoproteins were obtained by hot citrate buffer extraction followed by precipitation with ethanol (15). The precipitates were further purified by a concanavalin A-agarose column (1.0 × 10 cm), which was equilibrated with Tris-HCl buffer (pH 7.3) containing 0.2 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. The column was eluted by 0.1 M NaCl solution containing 0.1 M α-methyl-D-mannnoside. Since the elution profile was not changed by using 0.2 M α-methyl-D-mannnoside, we used 0.1 M concentration for the elution. The mannoprotein fraction was dialyzed and lyophilized.

Glycopeptidase A Digestion

Glycopeptidase A from sweet almond (Seikagaku Kogyo Co., Tokyo, Japan) is specific for release of *N*-linked oligosaccharides from glycoprotein or glycopeptide (16), whereas hydrazinolysis releases both *N*- and *O*-linked oligosaccharides from a glycoprotein (17). When the oligosaccharides released from cell wall mannoprotein by hydrazinolysis were analyzed on HPLC after fluorescence labeling, several extra by-products were observed, which were probably formed from the *O*-linked oligosaccharides by the chemical reaction (hydrazinolysis), because these by-products disappeared in samples from glycopeptidase A digestion. Hence, we used glycopeptidase A to release the *N*-linked oligosaccharides from total cell mannoprotein. Ten milliunits of glycopeptidase A was dissolved in 500 μl of 0.1 M sodium acetate buffer (pH 5.0). Each 3 μl of the above enzyme solution was added to samples containing 1 mg of mannoprotein in 10 μl of the same buffer. The mixture was incubated for 18 h at 37 °C, and the reaction was stopped by boiling for 5 min and centrifuged to remove insoluble material.

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectroscopy.

TABLE I
Purification of the external invertase synthesized by Δoch1 cells at 25 °C

One unit is defined as the enzyme activity that produces 1 μmol of glucose from sucrose for 1 min at 30 °C at pH 5.0.

Step	Total protein[a]	Total activity	Specific activity	Recovery
	mg	*units × 10³*	*units/mg*	*%*
1. Crude cell extract	5,600	76.5	13	100
2. Streptomycin sulfate	4,200	66.5	16	87
3. Ammonium sulfate	150	57.6	384	78
4. DEAE-Sephadex	9	33.3	3,700	44
5. Sephadex G-200SF	4.5	20.6	4,560	27

[a] Protein was determined according to Bradford (11) using bovine serum albumin as a standard for steps 1–4. Protein concentration for step 5 was determined from the absorbance at 280 nm ($\epsilon_{1\%}^{280\,nm} = 22.5$) (12).

Pyridylamination

The oligosaccharides released from proteins, either by hydrazinolysis or by glycopeptidase A, were labeled with 2-aminopyridine to facilitate the sensitive detection by fluorescence (18, 19). Pyridylamination was carried out by using the commercially available reagent kit (Takara Shuzo Co., Kyoto, Japan). The pyridylaminated derivatives (PA oligosaccharides) were loaded on a Toyopearl HW-40F column (1.0 × 40 cm) (Tosoh Co., Tokyo, Japan), which was prewashed with 10 mM ammonium acetate buffer (pH 7.0), and eluted with the same buffer to remove residual 2-aminopyridine. PA oligosaccharides were detected by fluorescence (excitation wave length (Ex) = 320 nm, emission wave length (Em) = 400 nm).

High Performance Liquid Chromatography (HPLC)

The separation of PA oligosaccharides was carried out by HPLC using a Shimadzu LC-6A chromatograph system equipped with a fluorescence spectromonitor RF-530.

Anion Exchange HPLC—Since some of *N*-linked oligosaccharides on invertase are phosphorylated in *S. cerevisiae* (20), anion exchange Cosmogel QA column (0.8 × 7.5 cm) (Nakarai Tesque, Kyoto, Japan) was used to separate neutral oligosaccharides from the phosphorylated ones. The column was previously equilibrated with 10 mM ammonium acetate buffer (pH 8.0) at a flow rate of 0.5 ml/min. At 20 min after a sample injection, the buffer concentration was increased linearly up to 45 mM for 15 min. The neutral oligosaccharide fractions were eluted within 17 min after the sample injection and pooled for further analysis. PA oligosaccharides were detected by fluorescence (Ex = 310 nm and Em = 380 nm).

Size-fractionation HPLC—The retention time of oligosaccharide largely depends on the number of sugar residues in the amine-modified column chromatography (21). Size-fractionation HPLC was performed with Asahipak NH2P-50 (0.46 × 25 cm) (Asahi Chemical Co., Tokyo, Japan) at a flow rate of 1.0 ml/min. Two solvents, A and B, were used. Solvent A was composed of 200 mM acetic acid adjusted with triethylamine (pH 7.3) and acetonitrile (35:65, v/v). Solvent B was composed of 200 mM acetic acid adjusted with triethylamine (pH 7.3) and acetonitrile (50:50, v/v). The column was equilibrated with solvent A. After the sample injection, the proportion of solvent B was increased linearly up to 100% for 50 min. PA oligosaccharaides were detected by fluorescence (Ex = 310 nm and Em = 380 nm).

Reverse-phase HPLC—The reverse-phase HPLC was performed with Cosmosil 5C18-P (0.46 × 15 cm) (Nakarai Tesque) at a flow rate of 1.0 ml/min. Two solvents, C and D, were used. Solvent C was 10 mM sodium phosphate buffer (pH 3.8), and solvent D was 10 mM sodium phosphate buffer (pH 3.8) containing 0.1% 1-butanol. The column was equilibrated with a mixture of solvent C and D (95:5, v/v). At 10 min after the sample injection, the ratio of solvent D was increased linearly up to 100% for 90 min. Samples were detected by fluorescence (Ex = 320 nm and Em = 400 nm).

α-1,2-Mannosidase Digestion

One microunit of α-1,2-mannosidase (from *Aspergillus saitoi*) (22) and 10 pmol of PA oligosaccharide were dissolved in 20 μl of 0.1 M sodium acetate buffer (pH 5.0). The reaction mixture was incubated for 18 h at 37 °C and then boiled to stop the reaction. After centrifugation, the supernatant was subjected to HPLC as described previously.

Partial Acetolysis

Partial acetolysis of PA oligosaccharide (23) was carried out to release α-1,6-linked mannose specifically. A 5-nmol sample of PA oligosaccharide was peracetylated with a mixture (40 μl) of acetic anhydride and pyridine (1:1) at 100 °C for 15 min in a sealed glass tube. After removing excess reagent by evaporation, 20 μl of a mixture containing acetic anhydride-acetic acid-sulfuric acid (10:10:1) was added. The tube was resealed and incubated at 37 °C for 15 h. Four microliters of pyridine was added, and the solution was dried by evaporation. Then 0.4 ml of saturated sodium bicarbonate solution was added to the residue, and the products were extracted five times with 0.3 ml each of chloroform. After evaporation of the combined chloroform extract, the sample was subjected to hydrazinolysis and *N*-acetylation as described, and the PA oligosaccharide sample was analyzed by HPLC.

FAB-MS

Fast atom bombardment mass spectroscopy (FAB-MS) was carried out using a JMS-HX110 mass spectrometer (JEOL Co., Tokyo, Japan) equipped with an FAB ion source operated with xenon at 10 kV of accelerating voltage. The matrix used was a mixture of nitrobenzyl alcohol and glycerol (6:4). Samples (1–2 μg of PA oligosaccharide) were desalted by HPLC using either an Asahipak NH2P-50 (0.46 × 25 cm) or a Tosoh TSKgel Amide-80 column (0.46 × 25 cm) at a flow rate of 0.5 ml/min with a mixture of acetonitrile and water (1:1).

High Resolution ^1H NMR

^1H NMR spectra of PA oligosaccharide were measured on a JNM-GX400 (JEOL Co.) at 50 °C. Samples (50–200 μg, except for 18 μg of authentic $Man_8GlcNAc_2$-PA) were dissolved in 99.96% D_2O and lyophilized. After three repetitions of the above procedure, the samples were finally dissolved in 500 μl of 99.996% D_2O. The chemical shifts (δ) are expressed in parts/million (ppm) downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but they were actually measured by reference to internal acetone (δ 2.217 ppm). Authentic PA oligosaccharides were purchased from Takara Shuzo Co.

Double and Triple Mutant Preparation

Double and triple mutants were constructed by sporulating the appropriate heterozygous diploids and isolating the haploid mutant clones after tetrad dissection (5). In cross between YS57-5C and LB1-10B, the sporulation ratio was about 10%, and the segregant carrying the *och1* mutation was not obtained. Thus, among them, YN1-28A and YN1-28B, the haploid clones carrying *mnn1* mutation were selected by the Western blot analysis using anti-α-1,3-mannan antibody (kindly supplied by Dr. R. Schekman, University of California, Berkeley) and these strains were crossed to the following Δ*och1* strains. Two strains, YN3-1D and YN4-18A, carrying Δ*och1 mnn1* double mutation, were obtained from the crosses between YN1-28A and YS57-5A, and between YN1-28B and YS57-2C, respectively. Three Δ*och1 alg3* double mutants, designated YN2-12A, YN2-14A and YN2-15C, were isolated from the cross between PRY90 and YS57-2C. The size of invertase from each clone was analyzed on SDS-PAGE (10) using 4–20% gradient gel (SDS-PAGE Plate 4/20, 10 cm × 10 cm, Daiichi Pure Chemicals) by Western blotting (4) using anti-invertase polyclonal rabbit antibody. The phenotypes of Δ*och1 mnn1* and Δ*och1 alg3* mutants were confirmed by analyzing the length of oligosaccharides released from cell wall mannoprotein by glycopeptidase A on size-fractionation HPLC. The Δ*och1 mnn1 alg3* triple mutant, YN5-2C, was constructed by the cross between YN3-1D (Δ*och1 mnn1*) and YN2-12A (Δ*och1 alg3*). The phenotype was confirmed by comparing the invertase size with those from each double mutant by Western blotting.

RESULTS

Comparison of Invertase Mobility between the Mutants—Fig. 1 shows the electrophoretic patterns of external invertases. The Δ*och1* cells gave a few bands at a faster migration position than the wild type cells, indicating a deficiency of polymannose outer chain. The invertase from Δ*och1 mnn1* double mutant showed a few discrete bands at a smaller size than that from Δ*och1* cells (Fig. 1, *lanes 2* and *3*). As demonstrated for the *mnn1 mnn2 mnn9* triple mutant (24), each ladder band in Δ*och1 mnn1* cells reflects the number of sugar chains attached to invertase protein, which has 13 potential *N*-glycosylation sites in wild type *S. cerevisiae* (25). The invertase from *alg3* cells gave a broad smear (Fig. 1, *lane 4*), the migration range of which is similar to that of wild type cells, indicating an addition of polymannose outer chain. The mobility of invertase from Δ*och1 alg3* double mutant cells is slightly faster than that from Δ*och1* cells (Fig. 1, *lanes 2* and *5*), suggesting a shorter oligosaccharide length in Δ*och1 alg3* cells than in Δ*och1* cells. Furthermore, the Δ*och1 mnn1 alg3* triple mutants produced a still smaller invertase molecule (Fig. 1, *lanes 3*, *5*, and *6*) as a result of accumulation of three independent mutations that affect *N*-linked oligosaccharide biosynthesis.

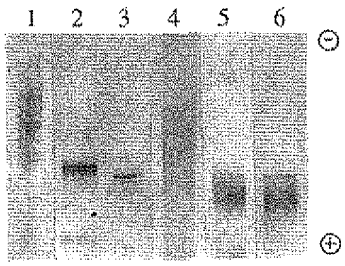

FIG. 1. **Comparison of electrophoretic mobility of external invertases on SDS-PAGE (10) using 4–20% gradient gel between the mutants EHA-1C (wild, *1*), YS52-1-1B (Δ*och1*) (*2*), YN3-1D (Δ*och1 mnn1*) (*3*), PRY90 (*alg3*) (*4*), YN2-1-2A (Δ*och1 alg3*) (*5*), and YN5-2C (Δ*och1 mnn1 alg3*) (*6*).**

N-Linked Neutral Oligosaccharide Length—The size of *N*-linked neutral oligosaccharides released by hydrazinolysis from the external invertase of Δ*och1* cells was analyzed by size-fractionation HPLC. The PA oligosaccharides were separated into three major peaks (*peaks 1–3*) with some other minor peaks (Fig. 2*A*). The elution time of peak 1 (21.8 min) is identical to that of authentic $Man_8GlcNAc_2$-PA (PA Sugar Chain 019, Takara). Judging from the elution time of PA glucose oligomers (Honen Oil Co.), peaks 2 (25.1 min) and 3 (28.3 min) were estimated to correspond to $Man_9GlcNAc_2$-PA and $Man_{10}GlcNAc_2$-PA, respectively. The Δ*och1* cells cultivated at the permissive temperature (25 °C) completely lacked the larger oligosaccharides that were observed in invertase from baker's yeast and from the *och1 ts* mutant under the permissive temperature (1).

The PA oligosaccharides released by glycopeptidase A from the cell wall mannoprotein of Δ*och1* cells showed a similar elution pattern on size fractionation HPLC, except for some minor peaks at 50–60 min (data not shown). Since the minor peaks were not observed in the PA oligosaccharide from external invertase of Δ*och1* cells, but were seen in the total mannoprotein from the *mnn9* mutant, these minor products may be derived from mannoprotein during the high temperature extraction of cell wall material.

The oligosaccharide from Δ*och1 mnn1* double mutant mannoprotein contained predominantly a single species, corresponding to $Man_8GlcNAc_2$-PA (Fig. 2*B*, *peak 4*), suggesting the loss of α-1,3-mannose addition due to the *mnn1* mutation. A minor component (10% of $Man_8GlcNAc_2$-PA) was observed at the elution time of 24.2–25.5 min, which corresponds to that of $Man_9GlcNAc_2$-PA (see "Discussion").

The elution pattern of PA oligosaccharide from Δ*och1 mnn1 alg3* triple mutant mannoprotein (Fig. 2*C*) reveals two major peaks (*peaks 5* and *6*). Judging from the elution time of PA glucose oligomers, peak 5 (12.3 min) corresponds to

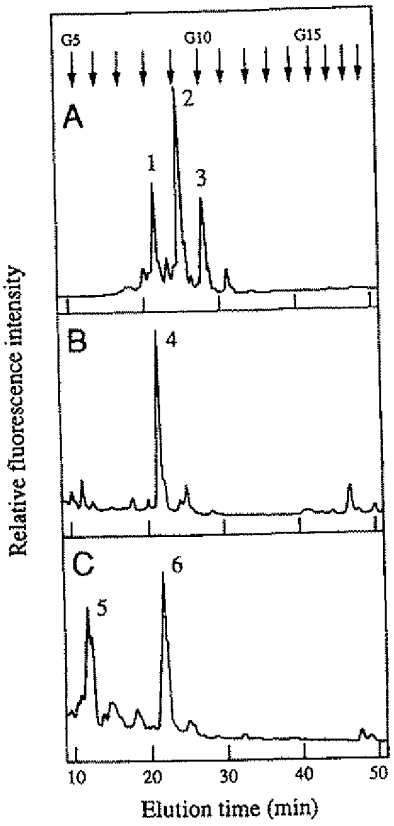

FIG. 2. **Elution profile on size fractionation HPLC of PA oligosaccharides released from YS52-1-1B (*Δoch1*) invertase by hydrazinolysis (*A*), YN3-1D (*Δoch1 mnn1*) mannoprotein by glycopeptidase A digestion (*B*), and YN5-2C (*Δoch1 mnn1 alg3*) mannoprotein by glycopeptidase A digestion (*C*).** Each sample is about 500 pmol. The elution times of authentic PA glucose oligomers are indicated by *arrows*.

$Man_5GlcNAc_2$-PA and peak 6 (21.8 min) corresponds to $Man_8GlcNAc_2$-PA. Most of these two peaks disappeared in *alg3* mutant cells, suggesting outer chain addition in the *alg3* single mutant (data not shown).

Oligosaccharide Structure of Invertase from Δoch1 Cells—Each PA oligosaccharide fraction corresponding to *peaks 1–3* in Fig. 2*A* from *Δoch1* cells was collected individually and analyzed on reverse-phase HPLC to examine its purity. Each showed a single peak on reverse-phase HPLC, suggesting a single component (data not shown). The elution time of component 1 (31.6 min) was in accordance with that of the authentic ER-form $Man_8GlcNAc_2$-PA sample (PA Sugar Chain 019, Takara). Components 1–3 were analyzed by positive ion FAB-MS to give positive molecular ion peaks at *m/z* 1,800, 1,962, and 2,123, respectively, which is consistent with the molecular mass of $Man_8GlcNAc_2$-PA (calculated 1,798.7), $Man_9GlcNAc_2$-PA (calculated 1,960.7), and $Man_{10}GlcNAc_2$-PA (calculated 2,122.8), respectively. These data confirmed the formation of PA derivatives and the estimation of each oligosaccharide length based on the elution time on HPLC.

α-1,2-Mannosidase removes only terminal α-1,2-linked mannose, and the size of each PA oligosaccharide component was analyzed on size-fractionation HPLC after the enzyme digestion. Component 1 was shifted from M8 ($Man_8GlcNAc_2$-PA) position to M5 position, the elution time of which is consistent with that of authentic $Man_5GlcNAc_2$-PA (PA Sugar Chain 017, Takara); component 2 was shifted from M9 position to M8 position; the elution time of component 3 was not shifted. The molecular mass of each digestion product was confirmed by FAB-MS. The M5 product derived from the M8 sample showed a protonated molecular ion at *m/z* = 1,313, and the M8 product from the M9 sample showed a protonated molecular ion at *m/z* = 1,800, indicating the structure of $Man_5GlcNAc_2$-PA (calculated 1,312.5) and $Man_8GlcNAc_2$-PA (calculated 1,798.7), respectively. These data were in accordance with the estimated structure of the component 1 as an ER-form core PA oligosaccharide. Based on the structure of component 1 component 2 was estimated to be a $Man_9GlcNAc_2$-PA in which one mannose residue, not connected by α-1,2 linkage, is attached to the $Man_8GlcNAc_2$-PA core oligosaccharide at the mannose of position 11 in Fig. 3*A*. In addition, component 3 was estimated as $Man_{10}GlcNAc_2$-PA in which one additional mannose is attached to the above $Man_9GlcNAc_2$-PA component at the mannose of position 9 in Fig. 3*A*.

The NMR spectrum for authentic $Man_8GlcNAc_2$-PA (ER-form core PA oligosaccharide, PA Sugar Chain 019, Takara) and those for $Man_8GlcNAc_2$-PA, $Man_9GlcNAc_2$-PA, and $Man_{10}GlcNAc_2$-PA samples from invertase of *Δoch1* cells are shown in Fig. 3 (*panels A–D*, respectively). Chemical shifts (δ, ppm) for C1 and C2 protons are summarized in Table II, and linkage assignments (Fig. 3 and Table II) were based on the reported chemical shifts (26, 27). The spectrum for the $Man_8GlcNAc_2$-PA sample from *Δoch1* cells (Fig. 3*B*) is identical to that for the authentic ER-form $Man_8GlcNAc_2$-PA (Fig. 3*A*). In the spectrum of the $Man_9GlcNAc_2$-PA sample, one additional C1-H signal was observed at 5.14 ppm, which is characteristic for the anomeric proton of the unsubstituted α-1,3-linked mannose (Fig. 3*C*). The signal at 5.14 ppm was doubled in intensity in the spectrum for the $Man_{10}GlcNAc_2$-PA sample, indicating the attachment of one additional α-1,3-linked mannose residue to the $Man_9GlcNAc_2$-PA structure. Since the α-1,2-mannosidase digestion of $Man_9GlcNAc_2$-PA yielded $Man_8GlcNAc_2$-PA, this α-1,3 linkage must be the mannose at position 11 in Fig. 3*A*, confirming the structure of $Man_9GlcNAc_2$-PA as shown in Fig. 3*C*. Considering the resistance of the $Man_{10}GlcNAc_2$-PA sample to α-1,2-mannosidase digestion, the oligosaccharide can be assigned the structure shown in Fig. 3*D*. The oligosaccharide structures shown in Fig. 3 (*panels A–D*) are in good agreement with the chemical shifts assigned in Table II.

Oligosaccharide Structure of Mannoproteins from Δoch1 mnn1 Double Mutant—The major PA oligosaccharide obtained from *Δoch1 mnn1* cells on size-fractionation HPLC (*peak 4* in Fig. 2*B*) showed a single peak on reverse-phase HPLC, designated as component 4 (data not shown). The elution time of this component 4 was identical to that of authentic $Man_8GlcNAc_2$-PA and the molecular mass was confirmed by FAB-MS, which gave *m/z* 1,800, as estimated for $Man_8GlcNAc_2$-PA (calculated 1,798.7). After α-1,2-mannosidase digestion of component 4, the peak was shifted from M8 to M5 position on size fractionation HPLC. The NMR spectrum of this component 4 (Fig. 3*E* and Table II) was identical to that of authentic $Man_8GlcNAc_2$-PA (Fig. 3*A*). From these data, we conclude that the *Δoch1 mnn1* cells predominantly produced the ER-form $Man_8GlcNAc_2$ as a neutral oligosaccharide.

Oligosaccharide Structure of Mannoproteins from Δoch1 mnn1 alg3 Triple Mutant—Each PA oligosaccharide fraction, corresponding to *peaks 5* and *6* in Fig. 2*C*, from *Δoch1 mnn1 alg3* mutant cells showed a single peak on reverse-phase HPLC (components 5 and 6, respectively). The elution time on reverse-phase HPLC of component 6 was same as that of the authentic ER form of $Man_8GlcNAc_2$-PA. The ratio of components 5 and 6 was 1:1.4. It is reported that the *alg3 sec18* mutant produced external invertase containing $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ *N*-linked oligosaccharides after the temperature shift (28). In accordance with the reported oligosaccharide structure of *alg3 sec18* (28), we assumed that component 5 produced by *Δoch1 mnn1 alg3* mutant cells must be the

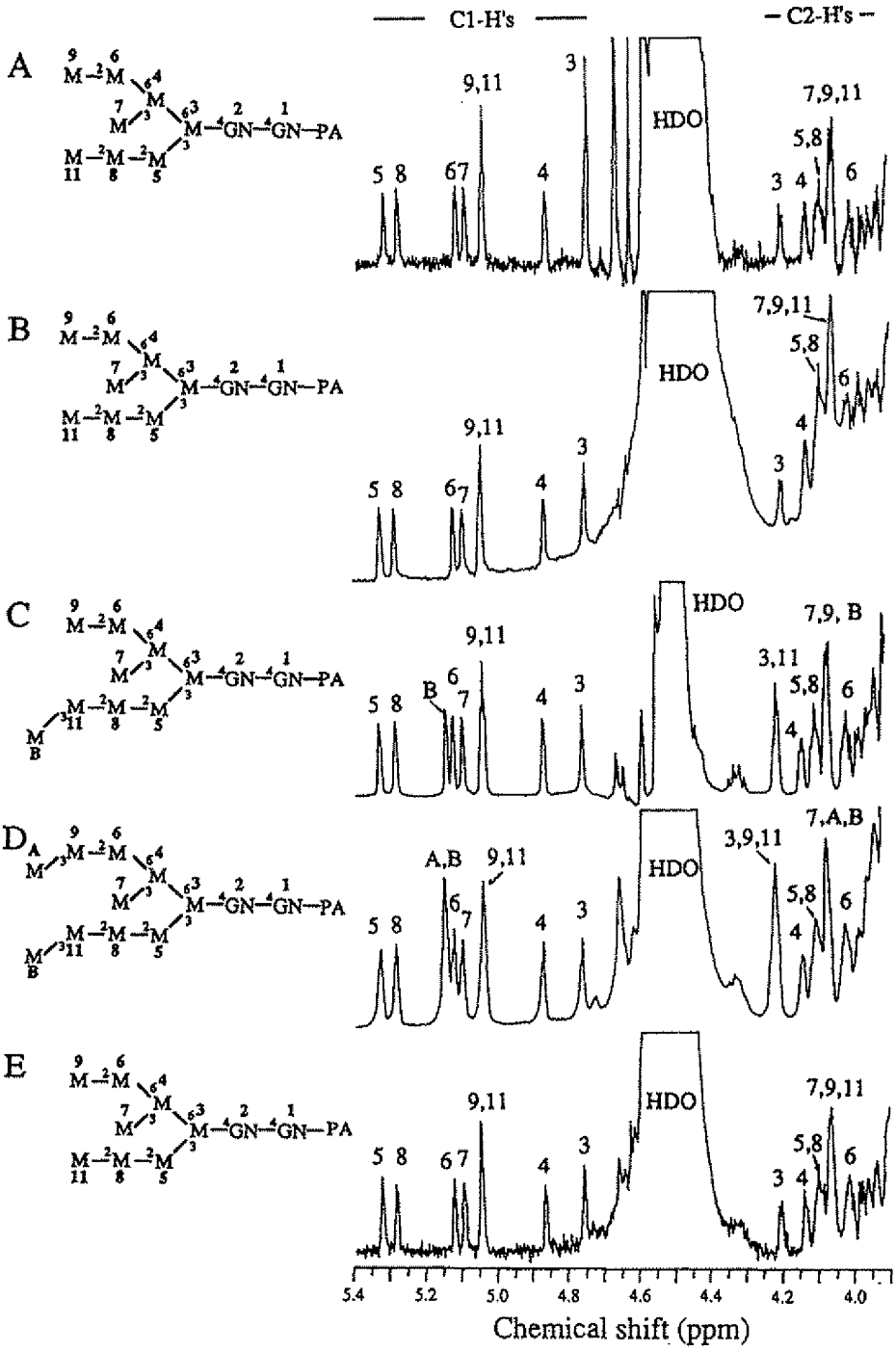

FIG. 3. **Comparison of the 400 MHz ^{1}H NMR spectra for purified neutral PA oligosaccharide fractions from Δ*och1* invertase (*B–D*) and from Δ*och1 mnn1* mannoprotein (*E*).** *A*, authentic ER-form core PA oligosaccharide, $Man_8GlcNAc_2$-PA, and PA oligosaccharide purified from the following: *B*, peak 1 in Fig. 2*A*; *C*, peak 2 in Fig. 2*A*; *D*, peak 3 in Fig. 2*A*; *E*, peak 4 in Fig. 2*B*. All signals in *spectrum B* are identical to those in *spectrum A*, whereas *C* and *D* have an additional anomeric proton signal at δ 5.14 equivalent to one and two protons, respectively, that is characteristic of the unsubstituted α-1,3-linked mannose.

$Man_5GlcNAc_2$-PA shown in Fig. 4*A*, and component 6 may be the ER-form $Man_8GlcNAc_2$-PA shown in Fig. 3*A*. To confirm these structures, components 5 and 6 were subjected to α-1,2-*mannosidase digestion and partial acetolysis*. After α-1,2-mannosidase digestion, the component 5 peak was shifted from the M5 position (12.0 min) to the M3 position (7.2 min) (Fig. 4, *A* and *B*) on size-fractionation HPLC. The molecular masses of component 5 (M5) and enzyme digestion product (M3) were confirmed by FAB-MS, which gave *m/z* 1,313, as estimated for $Man_5GlcNAc_2$-PA (calculated 1,312.5), and *m/z* 989, estimated for $Man_3GlcNAc_2$-PA (calculated 988.4), respectively. After partial acetolysis, which specifically removes α-1,6-linked mannose residues, the peak of component 5 (12.0 min) was shifted to the M4 position (9.4 min) (Fig. 4, *C* and *D*). This M4 product showed a protonated molecular ion at *m/z* = 1,152, indicating the formation of $Man_4GlcNAc_2$-PA (calculated 1,150.4). These data confirmed the structure of component 5 as the $Man_5GlcNAc_2$-PA shown in Fig. 4*A*. In contrast, component 6 was shifted from the M8 position (21.5 min) to the M5 position (12.0 min) after α-1,2-mannosidase digestion, and from the M8 position (21.5 min) to the M4 position (9.4 min) after partial acetolysis (data not shown). The molecular mass of M5 product

TABLE II

^{1}H NMR chemical shifts of authentic ER-form core oligosaccharide ($Man_8GlcNAc_2$-PA) and PA oligosaccharides obtained from Δoch1 mnn1 cells

Residue	Authentic M8		Δoch1 M8		Δoch1 M9		Δoch1 M10		Δoch1 mnn1 M8	
	Shift	Intensity	Shift	Intensity	Shift	Intensity	Shift	Intensity	Shift	Intensity
	ppm		*ppm*		*ppm*		*ppm*		*ppm*	
C1-H										
3	4.748	1	4.753	1	4.755	1	4.753	1	4.753	1
4	4.863	1	4.867	1	4.867	1	4.863	1	4.863	1
5	5.323	1	5.322	1	5.328	1	5.324	1	5.322	1
6	5.115	1	5.120	1	5.118	1	5.116	1	5.118	1
7	5.091	1	5.094	1	5.096	1	5.091	1	5.092	1
8	5.283	1	5.283	1	5.283	1	5.279	1	5.283	1
9	5.039	1	5.043	1	5.038	1	5.030	1	5.043	1
11	5.039	1	5.043	1	5.038	1	5.030	1	5.043	1
A							5.141	1		
B					5.140	1	5.141	1		
C2-H[a]										
3	4.195		4.199		4.205		4.205		4.200	
4	4.131		4.131		4.132		4.130		4.131	
5	4.092		4.092		4.095		4.095		4.092	
6	4.005		4.010		4.009		4.012		4.008	
7	4.060		4.059		4.060		4.061		4.059	
8	4.092		4.092		4.092		4.095		4.092	
9	4.060		4.059		4.060		4.205		4.059	
11	4.060		4.059		4.205		4.205		4.059	
A							4.061			
B					4.060		4.061			

[a] Intensities for individual signals of C2-H could not be calculated due to the overlap of signals.

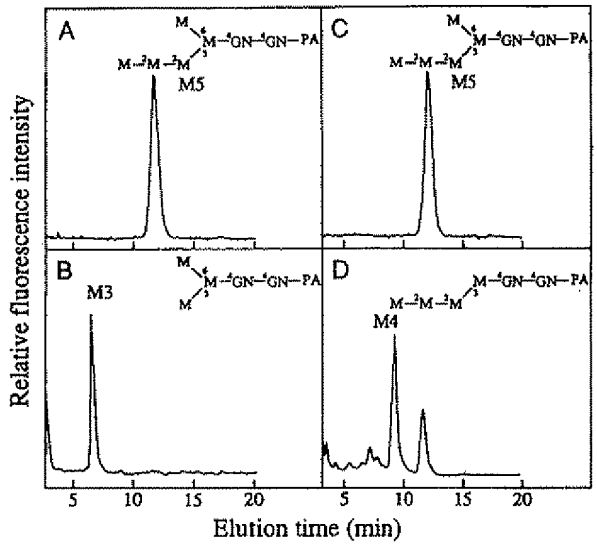

FIG. 4. **Elution profile on size fractionation HPLC of PA oligosaccharides released from Δoch1 mnn1 alg3 mannoprotein.** *A* and *C*, component 5 corresponding to peak 5 in Fig. 2*C*; *B*, after α-1,2-mannosidase digestion; *D*, after partial acetolysis.

from M8 sample after α-1,2-mannosidase digestion and that of M4 product from M8 sample after partial acetolysis were also confirmed by FAB-MS (data not shown), demonstrating the ER form of $Man_8GlcNAc_2$-PA (Fig. 3*A*) as component 6 (Fig. 2*C*).

DISCUSSION

We have determined the structures of the *N*-linked neutral oligosaccharides attached to invertase in Δ*och1* cells, as well as those of the neutral oligosaccharides attached to total mannoprotein from Δ*och1 mnn1* and Δ*och1 mnn1 alg3* mutants. The results demonstrate that the *OCH1* gene disruption leads to loss of function to add the α-1,6-polymannose outer chain to the ER-form core oligosaccharide. Previously (4), we reported that the *OCH1* gene encodes a novel membrane-bound mannosyltransferase that transfers mannose from GDP-[^{14}C]mannose to the oligosaccharide isolated from mannoprotein from Δ*och1* cells. At that time, we assumed that the OCH1 protein might be functional not in the initiation of α-1,6-polymannose addition to $Man_8GlcNAc_2$, but rather in the elongation of outer chain from $Man_9GlcNAc_2$, a conclusion that was based on the accumulation of a major PA oligosaccharide corresponding to $Man_9GlcNAc_2$-PA and the lack of mannose transfer to the "$Man_8GlcNAc$" in an *in vitro* reaction (4). The present results, however, are not consistent with the previous assumption, because all of the oligosaccharides identified in Δ*och1* cells lack α-1,6-mannose attached to the $Man_8GlcNAc_2$ core oligosaccharide. Therefore, we have examined whether the OCH1 protein can use $Man_8GlcNAc_2$-PA and $Man_9GlcNAc_2$-PA as mannose acceptors. The microsomal membrane fraction prepared from OCH1 protein-overproducing cells was found to transfer mannose efficiently to $Man_8GlcNAc_2$-PA to form $Man_9GlcNAc_2$-PA and to $Man_9GlcNAc_2$-PA to form $Man_{10}GlcNAc_2$-PA, and this *in vitro* reaction was diminished in the membrane fraction from Δ*och1* cells.[2] In addition, in the previous study (4), we used $Man_8GlcNAcOH$ (octamannosyl-*N*-acetylglucosaminitol) as acceptor, which was incorrectly called $Man_8GlcNAc$ (octamannosyl-*N*-acetylglucosamine). Now, we have confirmed that $Man_8GlcNAcOH$ does not serve as an acceptor for the OCH1 protein-dependent mannose transfer reaction, whereas $Man_8GlcNAc$ does. Accordingly, it now becomes clear that the *OCH1* gene encodes an α-1,6-mannosyltransferase that is functional in the initiation of mannose outer chain addition to the ER-form core oligosaccharide, as shown in Fig. 5. Romero and Herscovics (29, 30) reported the purification and characterization of α-1,6-mannosyltransferase, which is certain to be the same enzyme encoded by *OCH1* gene. Although we cannot exclude the possibility that the OCH1 protein may also function in the subsequent elongation to form a polymannose outer chain, this seems unlikely because reaction products larger than one mannose addition were not observed in our *in vitro* mannose transfer experiments even with a prolonged incubation period.[2]

Addition of α-1,3-mannose is known to occur with the core-like oligosaccharide to which mannobiose (Man-α-1, 2-Man-α-1,6-) is added to the ER-form oligosaccharide (compound E in Fig. 5), in wild type invertase (26), carboxypeptidase Y (31), and

[2] K. Nakayama, Y. Nakanishi-Shindo, and Y. Jigami, manuscript in preparation.

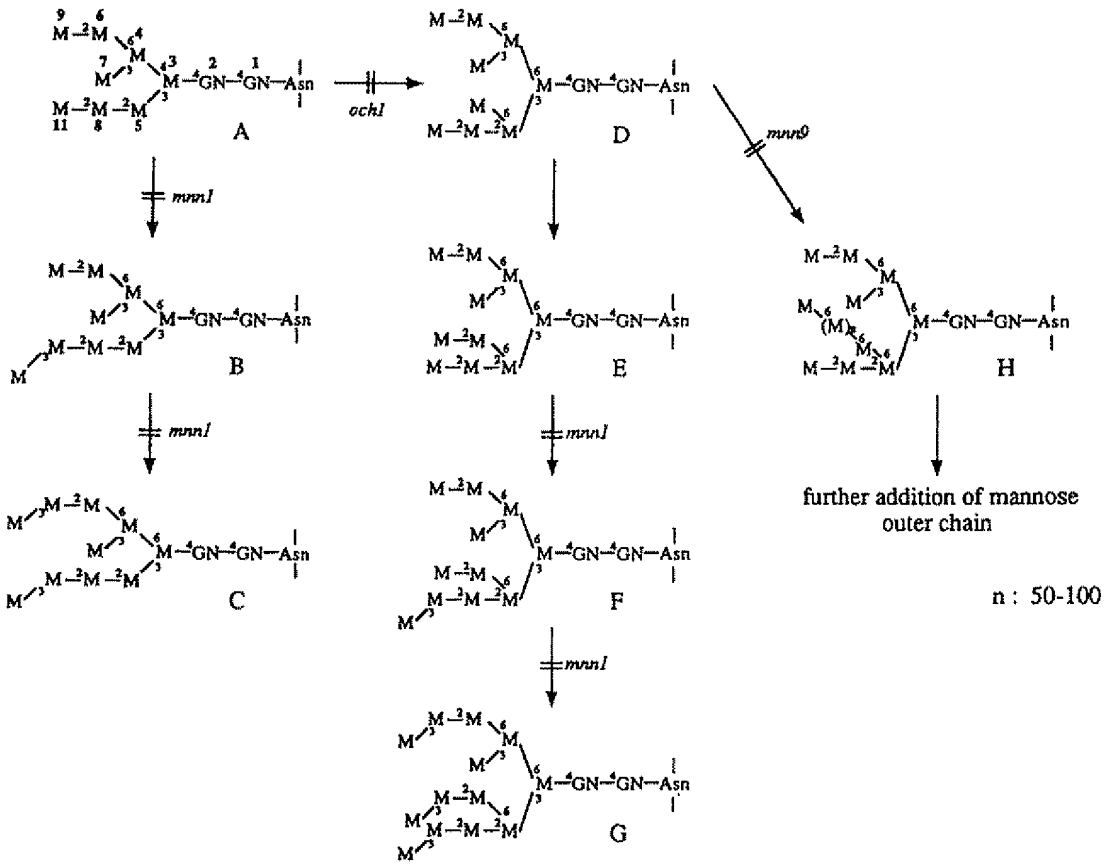

FIG. 5. ***N*-Linked oligosaccharide biosynthesis in *S. cerevisiae* and deficient steps caused by the *och1* and *mnn1* mutations, together with the proposed steps caused by the *mnn9* mutation (31).**

mnn9 mannoproteins (32) to form compounds F and G in Fig. 5. It is noteworthy that the α-1,3-mannose addition to the core oligosaccharide occurs even in the absence of this mannobiose unit (see compounds B and C in Fig. 5). The first α-1,3-mannose addition occurs at the terminus of the α-1,3-linked mannotriose branch (position 11 of compound *A* in Fig. 5), and the subsequent α-1,3-mannose addition occurs at the end of α-1,6-linked mannotetraose branch (position 9 of compound A in Fig. 5), which is consistent with the previous results for the oligosaccharides from both wild type and *mnn2 mnn9* mannoproteins (26, 32). This order of α-1,3-mannose addition to the core oligosaccharide may suggest the higher affinity of α-1,3-mannosyltransferase encoded by *MNN1* gene (33) to add the mannose at position 11 of compound A in Fig. 5 rather than at position 9 of compound A in Fig. 5.

We have clarified that the Δ*och1 mnn1* mutant cells produce predominantly the $Man_8GlcNAc_2$ core oligosaccharide due to the lack of α-1,3-mannosyltransferase activity, although about 10% of the oligosaccharide was observed at the elution time corresponding to the M9 PA oligosaccharide on size-fractionation HPLC (Fig. 2*B*). This fraction consisted mainly of two isomers. From the results of reverse-phase HPLC and α-1,2-mannosidase digestion (data not shown), one appears identical with the $Man_9GlcNAc_2$-PA from Δ*och1* invertase and the other is estimated as the M9 structure in which one hexose is attached to the mannose at position 11 of compound A in Fig. 5. The former isomer suggests some residual α-1,3-mannosyltransferase activity in Δ*och1 mnn1* mutant cells owing to a leaky phenotype of the *mnn1* mutation. This may be resolved by oligosaccharide analysis of Δ*och1* Δ *mnn1* double disruptant. The latter isomer was shifted from M9 position to M8 position after α-1,2-mannosidase digestion. It is possible that the $Glc_1Man_8GlcNAc_2$-PA was formed as the M9 structure due to either the escape from glucosidase trimming or the reglucosylation of glucose-free core oligosaccharide, but further structure analysis was not performed. Regardless, we believe that the Δ*och1 mnn1* strain is a novel host cell in which to produce a glycoprotein containing an ER-form oligosaccharide that is identical to the mammalian high mannose type oligosaccharide and that can function as a precursor to form a complex type sugar chain in mammalian cells by using recombinant DNA technology.

The Δ*och1 mnn1 alg3* cells accumulated both the $Man_8GlcNAc_2$-PA ER-form core oligosaccharide (Fig. 3*A*) and $Man_5GlcNAc_2$-PA oligosaccharide (Fig. 4*A*). The *alg3* mutant is known to accumulate lipid-linked $Man_5GlcNAc_2$ before transfer to the Asn residue of protein (8, 34). It is likely that the $Man_5GlcNAc_2$-PA, which lacks both the Man-α-1,2-Man-α-1,6-linked mannobiose and the Man-α-1,3-linked mannose of compound A in Fig. 5, was caused by transfer to proteins of the incomplete lipid linked $Man_5GlcNAc_2$. The formation of $Man_8GlcNAc_2$ is assumed to be caused by a leaky phenotype of the *alg3* mutation, as reported for the oligosaccharide structure from *alg3 sec18* invertase (28). The molar ratio of $Man_5GlcNAc_2$ to $Man_8GlcNAc_2$ was 1:1.4 in Δ*och1 mnn1 alg3* mutant, whereas it was 5:1 in *alg3 sec18* mutant. This may reflect the different growth conditions, because Δ*och1 mnn1 alg3* mannoprotein was obtained from cells cultivated at 28 °C, whereas invertase from *alg3 sec18* cells was obtained by a 3-h de-repression at 37 °C after cultivation at the permissive temperature due to the *ts* phenotype of the *sec18* mutant (28). This suggests that the accumulation of the incomplete precursor of *N*-linked oligosaccharides may be temperature-dependent in the *alg3* mutant, although the *alg3* mutant itself is not *ts* for cell growth

(8). The formation of mannose outer chain in the *alg3* mutant (Fig. 1, *lane 4*) was diminished in Δ*och1 mnn1 alg3* cells (Fig. 1, *lane 6*), which suggests that the OCH1 protein is functional in the initiation of outer chain elongation of $Man_8GlcNAc_2$ as well as $Man_9GlcNAc_2$. The *in vitro* experiment supports this assumption by demonstrating that extracts from OCH1 protein-overproducing cells have an activity to transfer mannose to $Man_8GlcNAc_2$-PA to form $Man_9GlcNAc_2$-PA.[2] In conclusion, our results suggest that the *OCH1* gene encodes an α-1,6-mannosyltransferase that is functional in the initiation of α-1,6-polymannose outer chain addition to the *N*-linked core oligosaccharide ($Man_8GlcNAc_2$ and $Man_9GlcNAc_2$) in yeast. Further studies on the substrate specificity and kinetic properties of OCH1 protein are in progress.

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REFERENCES

1. Nagasu, T., Shimma, Y., Nakanishi, Y., Kuromatsu, J., Iwama, K., Nakayama, K., Suzuki, K., and Jigami, Y. (1992) *Yeast* **8,** 535–547
2. Esmon, B., Esmon, P. C., and Schekman, R. (1984) *J. Biol. Chem.* **259,** 10322–10327
3. Ballou, L., Alvarado, E., Tsai, P.-K., Dell, A., and Ballou, C. E. (1989) *J. Biol. Chem.* **264,** 11857–11864
4. Nakayama, K., Nagasu, T., Shimma, Y., Kuromatsu, J., and Jigami, Y (1992) *EMBO J.* **11,** 2511–2519
5. Sherman, F., and Hicks, J. (1991) *Methods Enzymol.* **194,** 21–37
6. Ballou, L., Cohen, R. E., and Ballou, C. E. (1980) *J. Biol. Chem.* **255,** 5986–5991
7. Tsai, P.-K., Frevert, J., and Ballou, C. E. (1984) *J. Biol. Chem.* **259,** 3805–3811
8. Huffaker, T. C., and Robbins, P. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 7466–7470
9. Neumann, N. P., and Lampen, J. O. (1967) *Biochemistry* **6,** 468–475
10. Laemmli, U. K. (1970) *Nature* **227,** 680–685
11. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254
12. Trimble, R. B., and Maley, F. (1977) *J. Biol. Chem.* **252,** 4409–4412
13. Fukuda, M., Kondo, T., and Osawa, T. (1976) *J. Biochem. (Tokyo)* **80,** 1223–1232
14. Takasaki, S., Mizuochi, T., and Kobata, A. (1982) *Methods Enzymol.* **83,** 263–268
15. Peat, S., Whelan, W. J., and Edwards, T. E. (1961) *J. Chem. Soc.*, 29–34
16. Takahashi, N., and Nishibe, H. (1981) *Biochim. Biophys. Acta* **657,** 457–467
17. Kuraya, N., and Hase, S. (1992) *J. Biochem. (Tokyo)* **112,** 122–126
18. Kondo, A., Suzuki, J., Kuraya, N., Hase, S., Kato, I., and Ikenaka, T (1990) *Agric. Biol. Chem.* **54,** 2169–2170
19. Hase, S., Ibuki, T., and Ikenaka, T. (1984) *J. Biochem. (Tokyo)* **95,** 197–203
20. Smith, W. L., and Ballou, C. E. (1974) *Biochemistry* **13,** 355–361
21. Blanken, W. M., Bergh, L. E., Koppen, P. L., and Van den Eijnden, D H. (1985) *Anal. Biochem.* **145,** 322–330
22. Kobata, A., and Amano, J. (1987) *Methods Enzymol.* **138,** 779–785
23. Natsuka, S., Hase, S., and Ikenaka, T. (1987) *Anal. Biochem.* **167,** 154–159
24. Ballou, L., Gopal, P., Krummel, B., Tammi, M., and Ballou, C. E (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 3081–3085
25. Ziegler, F. D., Maley, F., and Trimble, R. B. (1988) *J. Biol. Chem.* **263,** 6986–6992
26. Trimble, R. B., and Atkinson, P. H. (1986) *J. Biol. Chem.* **261,** 9815–98 24
27. Cohen, R. E., and Ballou, C. E. (1980) *Biochemistry* **19,** 4345–4358
28. Verostek, M. F., Atkinson, P. H., and Trimble, R. B. (1991) *J. Biol. Chem.* **266,** 5547–5551
29. Romero, P. A., and Herscovics A. (1989) *J. Biol. Chem.* **264,** 1946–1950
30. Romero, P. A., and Herscovics A. (1992) *J. Cell. Biochem. Suppl.* **16D,** 175
31. Ballou, L., Hernandez, L. M., Alvarado, E., and Ballou, C. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3368–3372
32. Alvarado, E., Ballou, L., Hernandez, L. M., and Ballou, C. E. (1990) *Biochemistry* **29,** 2471–2482
33. Graham, T. R., Verostek, M. F., Mackey, V., Trimble, R., and Emr, S. D. (1992) *Yeast* **8,** S458
34. Orlean, P. (1990) *Mol. Cell. Biol.* **10,** 5796–5805